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CURRENT DATA ON MOLECULAR STRUCTURE OF CLOSTRIDIUM-BOTULINUM TY--ETC(U)
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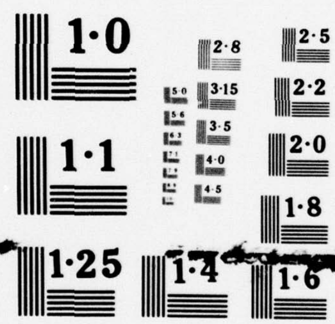
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TRANSLATION NO.: MUL 0549

11 17 Mar 78

12 19p.

6 TITLE: Current Data on Molecular Structure of Clostridium-Botulinum Types E and F

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REFERENCE: Zh. M. E. I. 52(12):22-28, 1975

DISTRIBUTION STATEMENT

Approved for public release;
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21 Trans. of Zhurnal Mikrobiologii, Epidemiologii i Immunogiologii (USSR) v52 n12 p22-28 1975.

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

Fort Detrick, Frederick, Maryland 21701

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Current data on molecular structure of Clostridium-Botulinum types E and F		5. TYPE OF REPORT & PERIOD COVERED Translation 6. PERFORMING ORG. REPORT NUMBER MUL 0549
7. AUTHOR(s) Ispolatovskaya, M.V. and L.N. Reshetnikova		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Zh. M.E.I. 52(12):22-28, 1975		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS USAMRIID Library, Ft. Detrick, Frederick, Md.		12. REPORT DATE 17 Mar 78 13. NUMBER OF PAGES 16
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release: distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Clostridium-botulinum type E
Clostridium-botulinum type F
Molecular structure

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

Current Data on Molecular Structure of Clostridium-Botulinum Types E and F

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pp. 16

From: Zh. M. E. I. 52(12):22-28, 1975

Cl. botulinum toxins are a cause of grave food intoxication. Up until now their mechanism of action has not been discovered. One of the approaches to the resolution of this significant problem must be the study of the structural and functional aspects of the toxic protein that possesses significant biological activity, which can be compared only with the catalytic action of ferments.

Over the many years of the study of botulism it became possible to classify the population of the stimulant into individual types producing toxins of various serological specificity, and to demonstrate that in various geographical zones the cause of illness is Cl. botulinum of various types: in the Western states of the USA -- type A, in the countries of Central and Western Europe -- type B, in Japan and Alaska the type E is an extremely frequent cause of botulism, in Denmark and California -- type F, etc. In the territory of the USSR most frequently the stimulants for botulism are types E and F /2/.

In recent years there have been published overviews by Borell et al. /13/ and by Schantz and Fugiyama /44/ in which information is presented concerning the type A and B toxins. In this connection we tried to illuminate the present state of the art regarding the molecular structure of the E and F type toxins.

Type E Cl. botulinum was first isolated in the USSR by Kushnir /7/ in 1934. The difference between this microorganism and ones previously known was substantiated by Gunnison et al. /23/. Significantly later, in 1958, in Denmark during an eruption of botulism there was identified a new type of Cl. botulinum -- the type F /25,33/.

Type E differs from other types in that it forms only a partially active toxin predecessor, or (in accordance with the new nomenclature) the progenitor /32/ whose activation is carried out by proteolithic ferments such as tripsine introduced from outside. The phenomenon of the activation of the type E toxin progenitor explains the seeming contradictions between the low toxicity of the cultures under laboratory conditions and the capability to cause fatal toxemia in humans during eruptions of botulism /17/.

A substantial amount of literature has been devoted to the study of the phenomenon of type E toxin activation by various proteolithic ferments. Still, the mechanism of the phenomenon is not fully clear. There are two known hypotheses on the mechanism of the fermentative activation of the progenitor of the type E toxin.

One of these is expressed by the researchers of the Sakaguchi school /39, 40/. According to this hypothesis, the action of the proteolithic ferments on the progenitor causes "molecular opening", i.e. a rupture of

the bonds between some amino acids. At the same time, there is no observable splitting-off of peptide fragments from the protein molecule. As a result, active areas that do not function in the structure of the native molecule of the progenitor become freed. In the opinion of the authors, the protoxin and the activated toxin have identical molecular weight and cannot be distinguished by amino acid content. Sacks and Covert /36/, Ispolatovskaya et al. /6/ have pointed out the similarity between the molecular sizes of the predecessor and the activated type E toxin.

In the opinion of a number of Canadian researchers /21/ the activation of the predecessor is conditional upon enzyme fragmentation of the protein molecule, in the course of which there takes place an unmasking of active areas that are concealed in the native protoxin molecule. The authors have indicated that the activation leads to the elimination from the toxin molecule of at least 18 amino acid residues. Thus, the activated toxin molecule has substantially lesser molecular weight.

Recent works by the American researchers Das Gupta and Sugiyama /14-16/ are of substantial interest to the study of the activation process. The authors showed a similarity in the molecular structures of type A, B and

E Cl. botulinum toxins, based on the presence of identical sub-units. In accordance to these data, tryptic activation includes includes the rupture of the molecule along two cysteine remnants that form an intra-chain disulfide bond, at least for 2 polypeptides with a molecular weight of 50,000 and 102,000 D. In contrast to other botulinic toxins, the type E toxin progenitor has a single-chain structure, and only after activation does in become a two-chain protein.

The role of proteinase in the activation of botulinic toxins was also studied /16/. Particular attention was devoted to a comparative study of the activation of a highly purified type E toxin progenitor. Trypsin and proteinase with trypsin-like specificity -- isolated from young cultures of a type B proteolithic strain of Cl. botulinum -- were used as activators. The degree of activation with the trypsin-like enzyme in all cases was lower than the degree of activation with trypsin. In the course of activation, both enzymes acted upon the peptide bonds which were formed by carboxyl groups of agrinin and lysin residues. The action of the trypsin was accompanied by a rupture of at least 2 bonds -- the lysin and the agrinin. The trypsin-like proteinase acted exclusively on the agrinin residue /14/. There are data on the possibility of trypsin activation of type F Cl. botulinum

cultures as well, in which case an increase in activity is observed only in young cultures 16-24 hours old /8/.

A number of authors have demonstrated a single antigenic and immunogenic activity of progenitor and toxin of type E Cl. botulinum /1, 3, 32/. Vorob'ev et al. first formulated the position concerning the unity of toxicity and antigenic activity centers of botulinic toxins. An analogous point of view is held by Boroff and Das Gupta /11/. In contrast to this, Kondo A. and Kondo S. /30/ expressed an opinion on the isolated nature of these centers, believing that the toxic center participates exclusively in the activation process. According to the data presented by Sugiyama and von Mayruaser /47/ the antigenic action of the activated toxin was somewhat lower than the protoxin activity. The authors believe that trypsin activation causes structural changes in the molecule during which additional toxic groups are liberated. This process does not affect antigenic activity.

In 1957 Gordon and Flock /22/, using the method of triple precipitation with ethyl alcohol in cold, obtained samples of purified type E Cl. botulinum with an activity of 1.9×10^7 LD_{50} per 1 mg general nitrogen and 8.5×10^4 LD_{50} per 1 mg general nitrogen for non-activated ones. The purification scheme devised by the authors was used subsequently by a number of researchers. In 1962 there

appeared a report by the Japanese researcher Schiokawa /45/ on the obtaining of crystallic Cl. botulinum type E toxin and protoxin. The preparations were homogenous during the study in the precipitation reaction in agaric gel with horse antiserum and during ultracentrifugation and had a specific activity of $5.5 \times 10^5 LD_{50}$ per 1 mg nitrogen for the prototoxin, and $5.0 \times 10^7 LD_{50}$ per 1 mg nitrogen for the toxin. In the subsequent years in accessible literature there has been no confirmation of the highly interesting results of this author. Vorontsov and Vasil'ev /4/ to purify the type E toxin proposed a method of fermentation based on the selective action upon proteins of proteolithic ferments. This method provided for a 33-100% exit of the toxin by Dlm, with an activity of $0.5 \times 4.0 \times 10^5$ Dlm/ml.

In this brief overview it is impossible to list all the works on purification of botulinic toxins, particularly since all these questions are examined in detail in the monograph by Vorob'ev et al. /3/. Here, particular attention is paid to the purification of partially harmless toxins and the study of the detoxification of the predecessor and the activated type E toxin.

The modern level of the development of molecular biology provides qualitatively different approaches to the study of macromolecules that are carriers of high biological

activity.

A substantial number of research works on obtaining highly purified preparation of botulinic toxin of the E type has been published by Canadian researchers. In 1964 Gerwing et al. /19/ reported on the isolation of type E toxin with low molecular weight (18,600 D) with an activity of 7.5 x 10^6 Dlm per 1 mg nitrogen. Analysis in an ultracentrifuge showed a precise symmetrical peak with a constant sedimentation of 1.7 S. The homogeneity of the preparation was substantiated also with electrophoresis.

Multifaceted research dedicated to isolating and studying the structure of type E and F botulinic structures has been carried out by the Japanese researchers Sakaguchi G. and Sakaguchi S. /37, 39/ using a number of modern methods for protein purification to isolate highly purified type E toxin samples with an activity of 4.0 to 10.0 x $10^5 LD_{50}$ per 1 mg nitrogen before activation, and 8.2 - 8.6 x 10^7 LD_{50} per 1 mg nitrogen after activation. The samples were homogenous when ultracentrifuged and had a similar sedimentation constant, equal to 11.5 S.

Based on previously developed purification methods, Kitamura et al. /26/ isolated a preparation called 12 S-predecessor. It was homogenous when processed in the ultracentrifuge, with electrophoresis and chromatography

at a pH of less than 6.0. During electrophoresis in an alkaline environment the authors observed its molecular dissociation into 2 protein components: the toxic E_{alpha} and the non-toxic E_{beta}. Each of these components had a sedimentation constant of $S_{20W} = 7.3$. In contrast to the beta-component isolated from the crystallic toxin of the A type, the E_{beta} component did not have the capacity to agglutinate erythrocytes. The sedimentation constant of the E_{beta} component of the predecessor was the same as the constant of the sedimentation of the alpha-component of the type A crystalline toxin. In 1969 the same authors demonstrated /28/ that E_{beta} and E_{alpha} may be more completely and quickly divided by chromatography into DEAE-sephadexe A-50. The molecular weight of the E_{alpha} and E_{beta} components according to gel filtration data is about 150,000 D.

The Japanese authors demonstrated the existence of the toxic protein Cl. botulinum type E in several molecular forms, determined their molecular and weight parameters, the toxicity of various forms of infection, regularities in the formation of antibodies to each component of the toxin's progenitor /41, 42/. The foundation for the listed research is the information obtained by Kitamura et al. /27/ concerning the existence of type E botulin toxin in at least 4 different forms: 12S and 7S activated by tryp-

sin, and 12S and 7S already subjected to such activation. At the XXI symposium on bacterial toxins in Japan there were interesting presentations containing data on the molecular structure of various types of botulinic toxins /31/: Cl. botulinum of all types form 12 S-toxins, called M-toxins, each of which contains one molecule of toxic and non-toxic components with an identical molecular size of 7 S.

Despite the almost 15 years' history of the study of the type F botulin toxin, the first reports of its isolation in highly purified state first appeared only in 1974-1975 /34, 35, 50/. The results of this research conflict somewhat. First of all, this is in connection with the molecular form of the isolated preparations.

On the basis of the theory of the unified structure of the natural toxins, isolated from the infection source, or rather the form that Lamanna and Sakaguchi /32/ called the progenitor-ancestor, Ohishi and Sakaguchi /34/ declare that they isolated a homogenous progenitor with a molecular weight of 235,000 D and an activity of 1.2 x 10^8 LD_{50} per 1 mg nitrogen and a sedimentation constant of 10.3 S. The type F toxin progenitor, as the type E toxin progenitor, contained no hemagglutinin.

Yang and Sugiyama /50/ do not share the point of view of the Japanese researchers and report on

isolating a neurotoxin with a molecular weight of 150,000 D. It is true that in 1975 Ohishi and Sakaguchi /35/ reported the molecular dissociation of the progenitor and on isolating a toxic component with a molecular weight of 128,000 D.

The causes for such a great activity on the part of botulin toxin are not clear. Attempts to explain the presence of toxicity with the features of the amino acid content were not successful, since botulin toxins of all types consist of the same amino acids as all the known proteins.

Schantz and Spero /43/, Spero /46/ attempted to determine the functional groups that are substantial to the mainfestation of biological activity by the botulinic toxins. The authors supposed that the mainfestation of the toxic properties of the botulin toxin is connected to the presence in its active centers of lysine ε-amino groups.

The results of research by Boroff and DasGupta /IO/ and Boroff et al. /12/ demonstrate the presence of tryptophane toxin in the molecule, which determines its biological activity. In the authors' opinion, tryptophane is either localized in the active molecules of the toxin molecule or assists their formation. It is important not only for manifestation of toxicity, but is at the same time a structural element of the antigenic determinant responsible

for the formation of protective antibodies /I2/. Gerwing et al. /2I/, who did not find type A and B tryptophane botulinic toxins in the molecules, believe that the decisive role in the manifestation of toxicity is played by cysteine. Beers and Reich /9/ asserted the role of free cysteine remnants in the manifestation of biological activity, but at the same time they do not consider these to be the basis for the action of the toxin.

When discussing reference data concerning the functional groups connected with toxicity it is first of all necessary to stress that they are contradictory. Evidently, toxicity is connected with all the amino acids that are included in the native tertiary structure of a protein. In other words, the blocking of each amino acid supporting the conformation characterstic of the given toxic protein leads to a decrease in toxicity. Proof of this can be found in the research by Knox et al. /29/ who came to the conclusion that the presence of reactogenic SH groups can affect the conformational stability of the botulinic toxin, without the direct participation by these groups in the structure of the active center.

Sugiyama et al. /48/ studied the dependence between the presence of disulfide bonds and biological activity by the botulinic toxins types A, E and F. Processing the toxins

with dithiotreithol causes a loss of 99% of the toxicity and a change in the molecular structure. On this basis the authors draw a conclusion on the significance of disulfide bonds in retaining the molecular structure that provides for manifestation of toxicity. When studying the dependence of immunogenity on the intactness of the disulfide bonds it was found that antitoxins obtained from toxins processed with dithiotreithol had a lesser antigenic and immunogenic activity. The authors stress that for manifestation of immunogenity it is necessary to retain at least one disulfide bond /49/.

Data on the size of the botulin toxin molecule of the E type are contradictory: 18,600 D and less (14,000 to 16,000 D) /19, 20/, from 86,000 to 12,000 D /36/, 200,000 D and greater /38, 39/, 350,000 and 150,000 D for the alpha fraction /28/. The difference between these figures is explained by the researchers by the different methods of purification used when isolating the toxins. In particular, Gerwing et al. /20/ believe that the data of Japanese authors that testify to the high molecular weight of type E toxin are erroneous and are based on a possible aggregation of the molecules during the purification.

In 1967 Schantz and Spero /43/, taking into account the suppositions of Gerwing et al. carried out a determination of molecular weight of native Cl. botulinum

cultures of all 6 types, using the method of sedimentation in an ultracentrifuge. The results showed that Cl. botulinum toxins of all the types studied were substances with a high molecular weight whose sedimentation constants corresponded to the following values: 19S for type A, 16S for type B, 14S for type C, 16S for type D, 14S for type E, 14S for type P. In the opinion of the authors, since there is no information available on the molecular weight of the toxin formed per se, it is not impossible that the polymerization of the toxin takes place within the bacterial culture. Notwithstanding, the Cl. botulinum toxins must have a relatively high molecular weight.

Attempts to obtain a toxin with a low molecular weight were made by Emodi and Lechowich /I8/. The authors reported isolating 2 toxic fractions of the type E toxin with a molecular weight of 9,000 and 5,000 D, respectively. In 1974 the research of Sacks and Covert /36/ was published, which also demonstrated the possibility of obtaining type E toxin with a low molecular weight. Studying the effects of the pH of the environment and purification methods on the molecular weight parameters of the type E toxin, the authors reported that they isolated at a pH of 4.5 a low-molecular toxic component with a molecular weight of 12,000 D. The possibility of molecular aggregation during purification was eliminated by chromatography on columns

with a sephadex of unpurified cultural filtrates. In the authors' experiments toxic activity was connected with fractions of 3 molecular weights: 12,000, 86,000 and 200,000 D and greater. Heimsh and Sugiyama /24/ were the first to demonstrate the possibility of type E toxin dissociation in an acid environment -- at a pH of 6.0. The authors were unable to find active fragments with low molecular weight.

Data on the size of type F botulin toxin molecules are presented by a number of authors. According to Schantz and Spero /43/ the sedimentation constant of native type F Cl. botulinum toxin is 14 S. In the work by Golshmid and Mikhaylova /5/ the molecular weight of type F toxin, determined by the method of filtration through a G-200 sephadex, was near 140,000 D. The molecular weight of the purified progenitor of the toxin found by Ohishi and Sakaguchi /34, 35/ equalled 235,000 D, and its toxic component's -- I28,000 D.

The neurotoxin isolated by Yang and Sugiyama /50/ had a molecular weight of 150,000 D. In connection with the introduction of new methods into laboratory research practice for the study of the antigenic and molecular structure of type E and F Cl. botulinum toxins it must be stressed that substantial success has been achieved in recent years.

For many years intensive and fruitful research has continued in Japan, the USA and Canada, where cases of botulism are basically caused by type E and F Cl. botulinum. Despite this fact, until recent times practically nothing has been known about the structure of the active center of the toxin or its mechanism of action. There is insufficient information on the connection between antigenic activity and toxicity. Reference data conflict on the subject of the molecular weights of a toxic protein. Therefore, to combat botulism it is necessary first of all to carry through a multifaceted study of the macromolecules of a toxic protein as one of the problems of modern molecular biology.

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